EXHIBIT 4

HUMAN GENE TRANSFER/THERAPY PROTOCOL

9110-010

Rosenberg, Steven A., National Cancer Institute; *Immunization of Cancer Patients Using Autologous Cancer Cells Modified by Insertion of the Gene for Tumor Necrosis Factor (TNF).*
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INITIAL PROPOSAL OF CLINICAL RESEARCH PROJECT

Date:_____Clinical Project Number:_____

To: Chairman, Clinical Research Subpanel, NCI

Recommended by: _____, Deputy Clinical Director, NCI

_____, Branch Chief(s)

Title: IMMUNIZATION OF CANCER PATIENTS USING AUTOLOGOUS CANCER CELLS MODIFIED BY INSERTION OF THE GENE FOR TUMOR NECROSIS FACTOR

Identifying Words: gene therapy, immunotherapy, interleukin-2, tumor infiltrating lymphocytes, tumor necrosis factor

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Estimated Duration of Study: 3 years

Number and Kind of Subjects Needed:	Number	Sex (M or F)	Age
Patients	50	M&F	18 & older

Precis: When tumor is resected from patients as part of the natural course of their treatment, an attempt will be made to establish a tissue culture line of the tumor. The gene coding for tumor necrosis factor will be introduced into these tumor cells and the integration and expression of this gene will be tested. Patients will become eligible for this study only if they develop metastatic cancer that has failed all standard effective treatment and have no other effective treatment options available to them. Tumor cells will be injected intradermally and subcutaneously into the thigh of these patients. The amount of tumor injected will be less than 1/50th the total tumor burden of the patient. In previous studies we have shown that these gene-modified tumor cells are more immunogenic than the native unaltered tumor. Attempts will then be made to grow immune lymphocytes either from the tumor site

Attempts will then be made to grow immune lymphocytes either from the tumor site or from the draining lymph nodes of these patients in order to use these lymphocytes for adoptive immunotherapy as detailed in previous protocols. The direct effect of the immunization with these immunogenic tumor cells will also be measured by assessing the impact on established tumor at other sites. Fifty patients receiving tumor inoculation will be included in this study.

I. OBJECTIVES

To evaluate the possible therapeutic efficacy of the injection of autologous cancer cells modified by insertion of the gene for tumor necrosis factor into patients with advanced cancer.

II. INTRODUCTION AND RATIONALE

1. Cell transfer therapy using TIL. Research in the Surgery Branch, NCI has been directed toward developing new immunotherapies for the treatment of patients with cancer (1-5). Based on extensive animal experimentation we developed treatment approaches using the administration of high dose interleukin-2 (IL-2) alone or in conjunction with the adoptive transfer of lymphokine activated killer (LAK) cells in patients with advanced cancer (1). We have now treated 178 patients using LAK cells in conjunction with IL-2 and 136 patients with high dose IL-2 alone (3). The results of the treatment of these 314 patients are shown in Table 1 and Table 2. These studies have demonstrated that the administration of high dose IL-2, either alone or in conjunction with LAK cells can result in the regression of advanced cancer in some patients. Approximately 10% of patients with metastatic renal cell cancer and melanoma will undergo a complete regression of cancer following treatment with LAK cells and IL-2 and an additional 10-25% of patients will undergo an objective partial remission.

In an attempt to improve upon these results, we identified and characterized a more potent type of killer cell called the tumor infiltrating lymphocyte (TIL) (6). These cells are cytolytic T lymphocytes. In both mice and human, TIL can develop the ability to specifically lyse the syngeneic or autologous tumor and not normal cells or allogeneic tumors (7,8). We demonstrated that TIL were from 50-100 times more potent on a per cell basis than were LAK cells in mediating the regression of established cancer in several murine tumor models (6). These animal experiments led to clinical trials of the use of TIL in humans (5). The results of the treatment of 50 patients with metastatic malignant melanoma are shown in Table 3. Thirty-eight percent of melanoma patients underwent an objective regression of their

cancer, however, the duration of responses has been variable and in many cases this response has been of short duration.

Extensive studies have been performed in the Surgery Branch, NCI to study the characteristics of human TIL and the mechanism of action of these cells in patients (9). In an attempt to study the traffic of TIL following cell infusion, 19 infusions of TIL labelled with Indium-111 were given to 18 patients and the distribution of these cells assessed using gamma camera imaging and sequential biopsies (10). Clear tumor localization of TIL was seen on 13 of 18 nuclear scan series and sequential biopsy data confirmed the homing of TIL to tumor deposits. These findings raised the possibility that TIL might be used as vehicles to deliver, to the tumor site, molecules that might enhance the antitumor activity of the TIL transfer.

To further study the distribution and survival of TIL we have performed studies of the retroviral transduction of the gene coding for neomycin resistance into TIL (11) and the subsequent infusion of these TIL into 10 autologous patients with advanced cancer (12). Extensive studies have been conducted on the first five patients and they are presented in Tables 4 and 5 and Figure 1. No toxicities of any kind could be attributed to the gene modification of the TIL. The expected toxicities associated with the concomitant administration of IL-2 were seen. Patients received up to 1.45 x 10^{11} gene transduced TIL populations. The percent of cells transduced in these populations varied between one and 11%. In all cases, integration and expression of the NeoR gene was demonstrated. As shown in Figure 1, gene modified TIL could be detected at tumor deposits as long as 64 days after gene transduction.

All safety studies performed in these patients showed no evidence of exposure to replication competent virus. 3T3 amplification and S+/L- assays revealed no replication competent virus present in the TIL at the time they were infused. Polymerase chain reaction analysis for the viral envelope gene and reverse transcriptase assay of the gene-modified TIL culture were also negative. Western blot analyses of patient serum at various times after cell

administration were all negative for evidence of exposure to virus as well. These studies demonstrated that gene modification of the TIL could be performed and that these TIL could be infused with no exposure of the patient to a replication competent virus. These studies provided us with valuable experience to perform subsequent studies of TIL modified with the gene for tumor necrosis factor (TNF). These studies began on January 29, 1991 and thus far two patients have been treated with escalating doses of TIL transduced with the gene for TNF. Thus far no side effects have been seen in these two patients.

2. Tumor necrosis factor. The injection of recombinant TNF can mediate the necrosis and regression of a variety of established experimental murine cancers (13-15). The combined administration of TNF and IL-2 mediated far greater antitumor effects against subcutaneous and liver tumors than either cytokine alone (16). The exact mechanisms of TNF antitumor effects are not clearly understood, although it appears that TNF has a significant effect on the vascular supply of tumors and CD8+ cells (15). Membrane-bound TNF may be involved in direct tumor lysis as well (17). These animal experiments have led to extensive tests of recombinant human TNF administered to humans with advanced cancer (3,18-21). In the Surgery Branch, NCI, we treated 38 patients with advanced cancer using escalating doses of recombinant TNF (supplied by the Cetus Corp., Emeryville, California) administered in conjunction with IL-2 (3). No antitumor effects of TNF administration have been seen in humans. However, when high local concentrations of TNF are achieved at tumor sites by direct intralesional TNF rejection, tumor regression in humans has been seen.

Extensive studies of the difference between the dramatic response of mice to the systemic injection of TNF and the lack of effect in humans have focused on the substantial differences in tolerance of mice and man to the administration of TNF. Tumor bearing mice can tolerate from 400-500 ug/kg TNF and these doses are required to mediate tumor regression; the administration of less TNF is far less effective (15). In contrast, the maximum tolerated

dose of TNF in both Surgery Branch, NCI and other studies is approximately 8 ug/kg/day. Thus when injected intravenously only 2% of the TNF dose required to mediate antitumor effects in the mouse can be administered to man.

Because of the unique effectiveness of TNF in the treatment of a variety of murine malignancies, we have sought means to selectively increase the local concentration of TNF at the tumor site. Because TIL can traffic directly to tumor deposits and concentrate at those sites we hypothesized that TIL transduced with the gene for TNF and producing large amounts of TNF in the local tumor microenvironment might have substantially increased antitumor effects compared to normal TIL. This hypothesis served as the basis for the protocol dealing with the administration of TIL transduced with the gene for TNF that is now underway.

In the course of these studies we used retroviral vectors to introduce the gene coding for TNF into murine and human tumors. These transduced tumor cell lines produced up to 15 ng TNF/10^6cells/24 hours. An example of the production of TNF over a two month period by high and low producer tumor lines from a sarcoma in C57BL/6 mice is shown in Figure 2. Extensive studies introducing the TNF gene construct into human melanoma cell lines were similar. These transduced cells contained one to two copies per cell of an unrearranged TNF vector genome and expressed transcripts homologous to the TNF cDNA corresponding to the proviral transcribed full length message (see Fig. 3). The human tumor cell lines produced up to 15 ng TNF/10^6 cells/24 hr.

Extensive experiments were performed to test the immunogenicity of these tumor lines in both syngeneic mice as well as in nude mice. In murine models we found that unmodified parenteral tumor cells grew aggressively when implanted subcutaneously into syngeneic mice, although tumor cells transduced with the TNF gene regressed in a significant number of animals after an initial phase of growth. An example of this phenomenon is shown in Fig. 4. This effect correlated with the amount of TNF produced and could be blocked with a specific anti-TNF antibody (see Fig. 5). The regression of these TNF producing cells was not associated with any demonstrable toxicity in the mice

bearing these tumors.

The increased immunogenicity of these TNF producing tumor cells was demonstrated by experiments showing the immunologic nature of this tumor regression. TNF producing tumor cells grew well in irradiated mice but not in non-irradiated mice. Further, the ability of tumor cells to regress was abrogated by depletion of the CD8+ T cell subset (see Fig. 6). Further, animals that experienced regression of TNF producing tumors rejected a subsequent challenge of unmodified tumor indicating the state of immunity to the tumor that had regressed. In addition, TNF producing tumor cells could function in a paracrine fashion by inhibiting the growth of unmodified tumor cells implanted at the same site (see Fig. 7). It thus appears from these murine studies that tumor cells elaborating high local concentrations of TNF could regress in the absence of toxicity in the host and that this regression was immunologically mediated.

Similar studies were performed using TNF gene modified human tumor cells injected into athymic nude mice. Following the injection of 4 to 8 x 10^6 human tumor cells into nude mice, non-transduced or neomycin transduced tumor cells grew progressively. However, tumors that were transduced with the TNF gene stopped growing after 8 to 10 days in all of the animals and complete tumor regression was seen in some of the mice (Fig. 8). In these experiments, DNA was extracted from some stable or regressing human tumors and Southern analysis revealed intact unrearranged proviral DNA present in all the regressing tumors induced by TNF-transduced cells. The proviral sequences were undetectable in the proliferating tumors formed by the non-transduced cells. The paracrine function of the TNF produced by human tumors was also seen. The localized elaboration of TNF by TNF transduced cells was also effective in suppressing tumor formation by control NeoR transduced cells injected at the same site.

It thus appears that in syngeneic mice the injection of TNF modified tumor cells was more immunogenic than unmodified cells and could induce an immunity sufficient to cause the regression of these TNF gene modified tumor

cells as well as normal tumor cells mixed at the same site. In this proposal we thus plan to take advantage of the increased immunogenicity of these TNF producing tumors to attempt to immunize patients with advanced and otherwise untreatable metastatic cancer. These tumor cells will be injected subcutaneously and intradermally into the thigh in an area that can be followed easily in an attempt to both immunize the patient against their cancer as well as to provide local immunization that could be used to grow lymphocytes for use in adoptive therapy. Lymphocytes will be obtained either from the draining inguinal lymph node group or from the tumor site itself.

In addition to the increased immunogenicity of the gene modified tumor, recent work in the Surgery Branch has suggested that the subcutaneous injection of tumor can lead to the development of more effective tumor infiltrating lymphocytes. In 12 successive experiments, TIL grown from visceral sites were simultaneously tested by careful <u>in vivo</u> titration against TIL from tumor injected into the cutaneous site. In 11 of 12 experiments, TIL from the subcutaneous location were more effective than those at visceral sites (see Table 6). In three other experiments, TIL from murine tumors in the liver were less effective than TIL from cutaneous sites.

In the human, other workers have shown that small cutaneous tumor auto inoculations can provoke significant immune responses. Hoover and Hanna have published work in which colorectal cancers from primary sites were irradiated and utilized for autologous immunization of patients in the adjuvant setting (22). Patients with Dukes B2 and C tumors demonstrated improved overall survival in a randomized study, presumably due to an effective immune response to the immunization. In addition, Berg and Mastrangelo have investigated the immunization of melanoma patients with irradiated autologous tumor, and have demonstrated the induction of significant T-cell infiltrates in tumors as well as rare clinical responses in patients with metastatic disease (23,24). These studies generally utilized irradiated tumor cell inoculation and therefore result in no tumor for the generation of TIL. The presence of tumor is vital for generating optimal TIL in that T-cells separated from fresh tumors will

grow in vitro with IL-2 but will show decreased in vivo efficacy if not re-exposed to tumor in culture (presumably due to the requirement of cultured T-cells for antigen exposure)(25). Furthermore, irradiated or non-viable tumor and tumor extracts produce immune responses in animals that are typically inferior to the responses to viable tumor (26). Therefore the injection of small amounts of viable tumor at a cutaneous site might not only result in tumor for TIL production, but also generate an immune response superior to that demonstrated using irradiated or non-viable tumor. This immune response may not only be seen at the tumor site, but pre-clinical models show that T-cells can be recovered in lymph nodes draining the site of tumor inoculation, which can be expanded in culture and show in vivo anti-tumor activity. This aspect of the cellular immune response to tumor immunization will be discussed later in the protocol.

Because tumor growth at the transplant site is necessary for TIL production, it is important to know if that will occur and what are the potential risks involved. Southam and Brunschwig inoculated a series of patients with a variety of metastatic cancers with their own resected tumors in graded doses (27,28). This revealed that the majority of patients could grow tumors at these inoculation sites if an adequate inoculum was administered. These were all patients with widely metastatic disease or unresectable advanced cancers and no impact from the inoculations on their overall clinical course was identified. For patients with widely metastatic cancer, a very small local cutaneous tumor inoculation (representing a fraction of their progressive metastatic disease) and subsequent resection of any growing tumor, is unlikely to significantly accelerate the course of their systemic disease. In support of this, one can cite the extreme case of tumor auto-inoculation which occurs when large numbers of malignant cells are intravenously infused as a result of peritoneal-venous shunting to palliate malignant ascites. Multiple clinical and post-mortem studies fail to show significant decreases in survival or alterations in the pattern of metastatic disease in shunted versus non-shunted patients (29-31). Such shunted patients

can develop microscopic metastatic implantation, but these studies show that these implants fail to reach a significant size prior to the patient's death from their pre-existing known metastatic disease. Certainly the cells which might escape from a small, cutaneously-implanted tumor site into the systemic circulation (if this occurs at all), would be far less than that from the intravenous auto-inoculation which occurs on a daily basis in these shunted patients or in any patient with widely metastatic cancer.

Thus, data suggests that auto-inoculation of a small amount of viable tumor at an isolated cutaneous site will often generate tumors for TIL growth, but that in the setting of widely metastatic cancer, such an approach is unlikely to significantly affect survival or the disease course of such patients. In the experiments in this protocol, however, the introduction of the TNF gene into tumor is designed to increase the immunogenicity of the tumor (as reviewed earlier) and will prevent tumor growth in most cases. The use of draining lymph node lymphocytes can thus provide a source of cells for use in adoptive immunotherapy of these patients.

Shu et al. have published data in murine models showing that draining lymph node lymphocytes (DLNL) from sites of tumor immunization can be sensitized in vitro (by mixed tumor-lymphocyte culture) and expanded in IL-2 (32-34). These cells can then be adoptively transferred to tumor-bearing mice and show antitumor activity (Table 7). Many of the features of these cells are similar to TIL (such as phenotype and tumor specificity patterns) although they have a lesser capability to expand in vitro and may be somewhat less effective on a cell-for-cell basis (25,35). These cultured draining lymph node cells are currently undergoing Phase I testing by Dr. Alfred Chang at the University of Michigan. In order to provide patients undergoing tumor immunization with a treatment alternative in the event of failure of the primary TIL culture, at the time of resection of inoculation sites (or at three weeks after inoculation if no tumor growth is apparent), draining lymph nodes will be excisionally biopsied to prepare an alternative T-cell culture for adoptive transfer. This will be performed with sensitization using

autologous cryopreserved tumor (from the original source used for inoculation) and IL-2. These lymph node lymphocytes will be given with systemic IL-2 exactly as intended for TIL.

III. PATIENT ELIGIBILITY

The eligibility of patients for whom cell lines will be established and who will be offered the treatment portions of this protocol are the same and are listed below:

1. Patients, age 18 or older, must have histologically confirmed metastatic cancer for which standard curative or palliative measures do not exist or are no longer effective. These patients have expected survivals of six months or less.

2. The estimated tumor burden of the patient must be at least 10 grams which is 50 times the weight of tumor cells used for the immunization.

3. Women of child bearing potential must have a negative pregnancy test.

4. Patients must have a negative HIV test.

5. Patients must have a performance status less than 2 and must be free of active systemic infections and other major medical illnesses of the cardiovascular and respiratory systems. They should have the following laboratory values:

   a. white blood cell count greater than 3000/mm^3

   b. platelet count greater than 150,000/mm^3

   c. bilirubin less than 1.7 mg/dl

   d. creatinine less than 1.7 mg/dl

6. Patients requiring steroid therapy will be excluded.

7. Patients who have received therapy with cytotoxic agents, steroids, other biologics, or radiotherapy in the 4 weeks prior to cell inoculation will be excluded.

IV. TREATMENT PLAN

1. Summary. Tissue culture lines will be established from resected tumors during the course of normal patient treatment. The gene for

tumor necrosis factor will be inserted into these tumor cells using ~~either~~ retroviral mediated gene transduction ~~or by transfection techniques using calcium phosphate or microinjection~~. Patients that develop advanced untreatable metastatic cancer and have failed all other effective treatments will be offered the possibility of joining this protocol. After signing an informed consent the patients will be registered in the protocol and will receive the injection of the gene modified tumor cells into the mid thigh. Up to 2 x 10^8 gene modified tumor cells will be injected in 1 ml subcutaneously and three centimeters lateral or vertical from this site the patient will receive two intradermal injections of 2 x 10^7 gene modified tumor cells in 0.1 ml each. These sites will then be carefully monitored. At three weeks the patient will undergo resection of several lymph nodes from the draining superficial inquinal area. These lymph nodes will be used to grow lymphocytes for adoptive cellular therapy of that patient. As predicted by animal models, it is not expected that tumor will grow at the local injection site. If, however, tumor does grow then the soft tissue in the area of the tumor injection will be resected when a tumor has reached one to two centimeters and an attempt will be made to grow tumor infiltrating lymphocytes from this site. Lymphocytes either from draining lymph nodes or from the tumor site itself (whichever become available first) will be grown in vitro by the standard techniques used in many previous protocols and assays published detail. These lymphocytes will then be adoptively transferred to the patient along with 720,000 IU/kg of IL-2, exactly as in our previous protocols and as previously published in detail. The impact of the immunization procedure on established tumor at other sites will also be monitored to evaluate whether this immunization procedure itself has anti-tumor effects. Biopsies of cutaneous or subcutaneous lesions may be performed.

2. Preparation of the TNF-NeoR vector containing supernatant. The TNF-NeoR vector used in this protocol is identical to that used in our approved protocol (#90-C-186) utilizing the introduction of these genes into human tumor infiltrating lymphocytes for use in human therapy. The following

description of the vector containing supernatant is taken verbatim from that approved protocol.

The TNF-NeoR vector was constructed by modifying the Moloney murine leukemia vector by techniques similar to those previously described. Retroviral vector supernatant is produced by harvesting the cell culture medium from the PA317 packaging line developed by Dr. A. Dusty Miller (36,37). This line has been extensively characterized and was used by us in our previous studies of the infusion of TIL modified by the LNL6 vector (11,12). The TNF-Neo vector preparations from PA317 will be extensively tested to assure that no detectable replication competent virus is present. Tests for replication competent virus will be conducted on both the vector supernatant and on the tumor cells after transduction. The vector includes the retroviral LTR promoting the TNF gene followed by the SV-40 early promoter and the gene coding for neomycin phosphotransferase. Testing will be the same as previously approved for the LNL6 supernatants used to introduce the NeoR gene into TIL (protocol 86-C-183c). The following tests will be run on the producer line and/or the viral supernatant:

1) The viral titer will be determined on 3T3 cells. Viral preparations with titers greater than 5 x 10^4 colony forming units/ml will be used.

2) Southern blots will be run on the producer line to detect the TNF gene.

3) TNF production by the producer line will be measured and should be significantly above baseline control values. TNF will be assayed using standard biologic assays on the L929 sensitive cell line (15) or by ELISA assay (R&D Systems, Minneapolis, MN).

4) Sterility of the producer line and the supernatant will be assured by testing for aerobic and anaerobic bacteria, fungus and for mycoplasma.

5) Viral testing will be performed including:

a. MAP test

b. LCM virus

c. Thymic agent

d. S+/L- assay for ecotropic virus

e. S+/L- for xenotropic virus

f. S+/L- for amphotropic virus

g. 3T3 amplification

6) Electron microscopy will be performed to assure the absence of adventitious agents.

The retroviral supernatant will not be used to transduce tumor cells injected into patients until approval is received from the Food and Drug Administration.

3. Preparation of gene-modified tumor cells. Tumor lines will be established in tissue culture from tumor fragments or single cell suspensions using standard tissue culture techniques (38). Tumor and normal tissue will be obtained immediately after surgery and processed as follows. The tumors were minced into 1 mm^3 fragments and dissociated with agitation in serum free DMEM (Dulbecco Modified Eagle Medium)(Biofluids) containing 2mM glutamine, 0.1 mg/ml hyaluronidase, 0.02 mg/ml Dnase I and 0.1 mg/ml collagenase for 3 hours at room temperature. The cell suspension was then centrifuged at 800 g for 5 minutes and the pellet resuspended in a culture medium consisting of 5ml of DMEM high glucose (4.5g/1) with penicillin and glutamine supplemented with 10% fetal calf serum. The cells were either centrifuged prior to being frozen in 90% FCS, 10% DMSO at -80° C, or plated in appropriate dishes or culture flasks in culture medium. Plated cells were incubated at 37° C in a humidified atmosphere of 5% CO_2 and 95% air. Within 48 hrs, the culture medium was changed in order to remove all non-attached material. Subsequently, cultures were incubated for a period of 6 to 8 days without medium change. The tumors grow as adherent monolayers in tissue culture flasks (Falcon #3028; 175 cm^2; 750 ml) containing about 50 ml of medium. When the cells are actively growing and not yet confluent the medium will be poured off and 30-50 ml of medium containing the retroviral supernatant with 5 ug/ml protamine will be added to

the flask (39). The flasks will be incubated at 37°C for six hours at which time the medium will be changed. This procedure will be repeated up to three times. After 24 to 48 hours medium containing 300 ug/ml G418 will be added directly to the flask and the cells will be grown and subcultured for 7 to 14 days in G418 containing medium. The G418 concentration may be raised to 1 mg/ml depending on the health of the culture.

4. Tests on the transduced tumor population. Following transduction, growth and selection of the tumor populations the following tests will be performed on the tumor prior to injection into patients.

1) Cell viability will be greater than 70% as tested by trypan dye exclusion.

2) Sterility will be assured by testing for aerobic and anaerobic bacteria, fungus and mycoplasma.

3) S+/L- assay must be negative.

4) Southern blot or PCR analysis will be run on the transduced tumor to assure that proviral sequences are present.

5) TNF protein assay to assure the production of TNF. Cells must be producing at least 100 pg TNF/10^6 cells/24 hours.

The S+/L- assay, the 3T3 amplification assay and the polymerase chain reaction assay are detailed in Appendix A.

5. Injection of tumor cells. Gene-modified tumor cells will be harvested from the culture flasks by exposure to 0.25% versene (EDTA) for 10 minutes. The cells will be washed three times by suspension in 50 mls normal saline and centrifugation. The final cell pellet will be suspended in normal saline and counted. 2 x 10^8 viable cells in 1 ml normal saline will be injected subcutaneously just beneath the skin in the anterior mid thigh and the overlying skin marked with a tatoo dot. If 2 x 10^8 cells are not available, fewer may be given but not less than 2 x 10^7 cells will be injected. About 3 cm lateral or vertical to this injection the patient will receive two intradermal injections (separated by 1 cm) of 2 x 10^7 gene modified tumor cells in 0.1 ml normal saline and these sites also marked by a

tatoo dot. These sites will be monitored weekly by a physician. At three weeks the patient will undergo excisional biopsy of superficial inguinal lymph nodes (without formal dissection) in the area draining the inoculation site for growth of lymphocytes. If tumor grows at any of these sites they will be excised when they reach 1 to 2 cm for growth of TIL. If no tumor growth is evident then the sites of tumor injection will be excisionally biopsied at 8 weeks after injection for pathologic analysis.

6. Growth of lymphocytes: The procedures used here are the same as those used in our previous protocols (40) involving the infusion of TIL (86-C-183) and is taken virtually verbatim from that protocol.

At least two days prior to surgery, peripheral blood lymphocytes are collected by leukapheresis for four hours. These are Ficoll-Hypaque separated and the mononuclear cells collected from the interface, washed in saline, and placed in culture in roller bottles at 10^6 cells/ml. Half are placed into AIMV (a serum free medium, Gibco Laboratories) with 6000 IU/ml IL-2 (Cetus), and half are placed into RPMI supplemented with 2% type-compatible human serum, penicillin (unless the patient is allergic), gentamicin, and 6000 IU/ml IL-2. After 3 to 4 days cells are centrifuged and the supernatants are collected and filtered. These are referred to as LAK supernatants.

Immediately upon resection of tumor or lymph nodes, the specimen(s) is transported to the laboratory in a sterile container and placed on a sterile dissection board in a laminar flow hood. A small representative portion is taken for pathologic analysis, and the rest is minced into pieces roughly 4 mm in diameter. These are placed into an enzyme solution of collagenase, DNAse type I, and hyaluronidase type V as previously described for overnight digestion at room temperature. The resulting suspension is filtered through a wire mesh to remove any large debris, washed in saline, and placed on Ficoll-Hypaque gradients. The interface containing viable lymphocytes is collected and washed in saline, and a portion is frozen for subsequent use as targets in cytotoxicity assays.

Lymphocyte cultures are initiated at 5 x 10^5 ml viable cells in 80%

fresh medium/20% LAK supernatant. For half the cells, the fresh medium is AIMV supplemented with penicillin,, fungizone, and 6000 IU/ml IL-2; for the other half, the fresh medium is RPMI supplemented with 10% human serum, penicillin, streptomycin, gentamicin, fungizone, and 6000 IU/ml IL-2. The cultures are placed into 6-well tissue culture dishes and incubated at 37° in humidified incubators with 5% CO_2. Cultures initiated from lymph nodes will be cocultivated with cryopreserved tumor stimulation cells at an initial ratio of at least 1:10 tumor cells to lymphocytes (but not more than 1:1) depending on the availability of tumor cells.

Usually the lymphocyte density is not much increased at the end of seven days in culture, and the cultures are collected, centrifuged, and resuspended at 5 x 10^5 total viable cells/ml in newly prepared 80%/20% medium mixtures of the same type. Occasionally a culture will have increased lymphocyte density and need medium replenishment prior to seven days. After this first passage, lymphocytes are subcultured by dilution when the density is between 1.5 x 10^6 and 2.5 x 10^6 cells per ml; densities of subcultures are established between 3 x 10^5 and 6 x 10^5 ml. Cultures are kept in 6-well dishes when the volume is less than 1 liter, and transferred to 3 liter polyolefin bags (Fenwal) when the volume reaches one liter. The subcultures from bags are accomplished with Fluid Fill/Weigh Units (Fenwal), which are programmed to pump prescribed weights of TIL culture and fresh medium into a new bag. When subculture volumes exceed 3 liters, the fresh medium used is AIMV. Cultures growing in serum-containing medium are thus diluted into AIMV, and no further LAK supernatant is added to cultures growing in serum-containing or serum-free medium.

If, during the growth of TIL, patients performance status has deteriorated to 3 or greater or if they have developed significant cardiac, renal, pulmonary or hematologic dysfunction than they will be taken off study and will not receive the infusion of TIL or IL-2.

When the total lymphocytes for a patient are ready for harvest, 5 x 10^6 cells are taken for cytological examination. Cytospins are examined for the

presence of remaining tumor. At least 200 cells are studied and therapy proceeds only when no tumor cells are found. Other lymphocyte samples are taken for characterization of cell surface markers and for assessment of cytotoxicity using techniques identical to that in our previous protocol (86-C-183; reference 23, attached). Briefly, lymphocytes are stained with fluorescent-labeled antibodies (Leu2, Leu3, Leu4, Leu7, Leu11, Leu5, Leu9, LeuM3, HLADDR and Tac). Chromium release assays are performed with K562, Daudi autologous tumor, and allogeneic tumor targets.

To infuse the lymphocytes they will be thawed and grown for one to three additional weeks using the same procedures detailed above. For infusion TIL are reharvested. At the time of cell collection, one liter of saline for injection is pumped through the collection chamber and the centrifuge is stopped. Lymphocytes are resuspended in the collection bag, the centrifuge is started again, and another liter of saline is pumped through to fully wash the free of tissue culture medium components. The cells are then filtered through a platelet administration set into 600 ml transfer packs (Fenwal), and 50 ml of 25% albumin and 450,000 IU of IL-2 are added to the 200 to 300 ml volume of cells in saline. The TIL are infused over 30 to 60 minutes through a central venous catheter.

7. Interleukin-2: The recombinant IL-2 used in this trial will be provided by the Division of Cancer Treatment, National Cancer Institute (supplied by the Cetus Corporation, Emeryville, CA)(28) and will be administered exactly as specified in our previously approved protocols (86-C-183c)(41). The IL-2 will be provided as a lyophilized powder and will be reconstituted with 1.2 ml/vial. Each vial contains approximately 1.2 mg of IL-2 (specific activity 18 x 10^6 IV/mg). Less than 0.04 ng of endotoxin are present per vial as measured by the limulus amebocyte assay. Each vial also contains 5% mannitol and approximately 130-230 ug of sodium dodecyl sulfate/mg of IL-2. Following reconstitution the IL-2 will be diluted in 50 ml of normal saline containing 5% human serum albumin and will be infused intravenously at a dose of 720,000 IU/kg over a 15 minute period every 8 hr, beginning from two

to 24 hr after the TIL infusion. IL-2 will be given for up to five consecutive days as tolerated. Under no circumstances will more than 15 doses of IL-2 be administered. The same toxicity criteria will be used as in our previous protocol (86-C-183c). Doses may be skipped depending on patient tolerance. Doses will be skipped if patients reach grade III or grade IV toxicity as detailed in Table 9. If this toxicity is easily reversed by supportive measures then additional doses may be given.

8. Concomitant therapy: Patients may receive concomitant medications to control the side effects of therapy (4,5). It is our plan to administer the same concomitant medications used in all previous TIL protocols. These include: acetaminophen (650 mg every 4 hours), indomethacin (50-75 mg every six hours) and ranitidine (150 mg every 12 hours) throughout the course of treatment. Patients may receive intravenous meperidine (25-50 mg) to control chills when they occur, although chills are unusual after the first one to two doses of IL-2. Hydroxyzine hydrochloride (25 mg every six hours) is given to treat pruritis. Steroids will not be used in these patients and if steroid are required, then the patient will immediately be taken off protocol therapy.

V. PATIENT EVALUATION

Parameters to be measured

	Pre	During Therapy								Week
	Study	D1	D3	D5	D6	D15	D17	D19	D20	7
Physical exam	X					X				
History	X	X								X
Performance Status	X									X
Assess for Tumor Effect[1]	X	X	X	X	X	X	X	X	X	X
Chemistry Survey[2]	X	X	X	X	X	X	X	X	X	X
Vital Signs	X	X	X	X	X	X	X	X	X	
Weight	X	X	X	X	X	X	X	X	X	X
CBC, Diff, Platelet	X	X	X	X	X	X	X	X	X	X
PT, PTT	X	X		X		X		X		X
FEV1, ABGs	X									
EKG	X							X		X
CXR	X				X	X		X		X
Cardiac Stress Test	X									
U/A and Culture	X									
Hb_sAg, HTLV III	X									
Brain CT or MRI	X									
Assess for adverse events status										X
PCR on PBL to detect NeoR gene	X	X	X	X				X		X
Tumor biopsy (if feasible)	X	X		X				X		X
Western blot (4070A envelope)	X									X
Serum assay for TNF	X	X	X	X	X	X			X	X

[1]To include assessment of all sites of disease.

[2]Includes total bilirubin, SGOT, LDH, Alkaline Phosphatase, Creatinine, Bun, CPK

1. Pretreatment (see Table on page 20).

a. Complete physical examination noting in detail the exact size and location of any lesions that exist.

b. Complete chemistry survey including electrolytes, liver function tests, calcium, magnesium, creatinine, BUN, CPK.

c. CBC differential count, PT, PTT, platelet count

d. Urine analysis and culture

e. Hepatitis screen

f. HIV titer

g. Pregnancy test if woman between the ages of 16 and 50

h. Chest x-ray

i. Electrocardiogram

j. Baseline x-rays and nuclear medicine scans to evaluate the status of disease.

k. CT scan or MRI scan of brain

l. 45 ml of clotted blood for serum storage and 45 ml of anti-coagulate blood for mononuclear cell cryopreservation. Selected patients may undergo pretreatment lymphocytapheresis.

m. Biopsy of tumor, if possible with minimal morbidity

2. During treatment. Patients will have a complete blood count and chemistry analysis panel at least every other day and a chest x-ray performed each week during treatment.

During the infusion of the lymphocytes, patients will be monitored closely in the Surgical Intensive Care Unit. Vital signs including blood pressure, pulse, and respirations will be measured every 15 minutes during the cell infusion and every 30 minutes for at least four hours or until the patient is stable. A pulse oximeter will be used for on-line measurement of oxygen saturation during and for the four hours after cell infusion as well. If the systolic blood pressure drops below 80 mm/Hg, or the oxygen saturation drops below 90% during the cell infusion, the cell infusion will be terminated immediately.

3. Post-treatment (see Table on page 20). Complete evaluation of evaluable lesions with physical examination, biopsy, if feasible, and appropriate x-rays and/or scans prior to each cell infusion cycle and at approximately eight weeks after the end of treatment to evaluate response to treatment.

Western blot analysis of patient serum to determine possible exposure to retrovirus envelope proteins will be performed at 7 to 8 weeks after treatment.

4. Criteria for response. Complete response is defined as the disappearance of all clinical evidence of disease for at least four weeks. Partial response is defined as the 50% or greater decrease of the sum of the products of perpendicular diameters of all lesions lasting at least four weeks with no increase in existing lesions or appearance of new lesions. Any patient having less than a partial response is considered to be non-responsive to treatment.

VI. POTENTIAL SIDE EFFECTS AND REPORTING OF ADVERSE REACTIONS.

1. Adverse drug reaction reporting will be performed in accord with NCI current reporting requirements for Phase I studies as follows:

Report by telephone to IDB within 24 hours (301-496-7957, Available 24 hours).

a. All life threatening events (Grade 4) which may be due to drug administration.

b. All fatal events.

c. The first occurrence of any previously unknown clinical event (regardless of Grade).

Written report to following within 10 working days to:

Investigational Drug Branch

P.O. Box 30012

Bethesda, Maryland 20824

2. Data will be submitted to CTMS at least once every two weeks. The NCI/DCT Case Report of ACES will be used to report to CTMS. All adverse

reactions should also be reported to the IRB.

3. Side effects of IL-2. A variety of side effects have been associated with IL-2 administration. We have had experience with the use of high-dose IL-2 either alone or in combination with cells or other cytokines in 1,039 courses in 652 patients. A listing of the side effects and their incidence is presented in Table 8.

TNF side effects including fever, chills, hypotension, oliguria, weight loss, nausea, vomiting and malaise.

All side effects will be graded using the standard toxicity sheet used in all prior IL-2 related protocols presented in Table 9.

4. Potential risks from injection of live tumor. These patients will receive the injection of approximately 0.1 gram of tumor into the skin or subcutaneous tissue of the anterior thigh. It should be emphasized that patients in this protocol will have histologically confirmed cancer with estimated tumor burdens of at least 10 grams or greater which is 50 times the amount of the tumor cells used for these immunizations. Based on our experimental data it is unlikely that these gene modified tumor cells will grow. These sites will be carefully monitored, however, and if tumor growth does occur then this site has been selected so that it could be widely excised with minimum morbidity. A small chance does exist, however, that if this site does grow that it might lead to spread of the injected tumor cells to draining lymph nodes or other sites in the body. The patients included in this protocol, however, will have metastatic cancer with limited life expectancy. The spread of the injected tumor is considered unlikely and in this patient population is unlikely to negatively influence the prognosis from their disease.

If the gene modified tumor cells do grow and produce TNF, then patients will be exposed to this systemic TNF. The maximum amount the TNF prod by the transduced tumor lines is about 15 ng/10^6 cells/24 hrs. If the t grow at all then they will be allowed to grow to a maximum of abou cells (or a 2 cm nodule) before they are excised. 2 x 10^9 cells

[08]

79

about 30 ug of TNF for 24 hours. We and others have previously shown that 70 kg patients can tolerate approximately 600 ug TNF iv every 24 hours (8-10/ug/kg). Thus for a 70 kg human, the amount of TNF being produced by these gene modified cells would be about 1/50th the amount of TNF already shown to be well tolerated by patients. It should be emphasized, however, that it is not expected that these tumor cells will grow based on experimental animal models. Further, if patients do develop signs of toxicity due to TNF exposure then it will be possible to excise the local nodules in the anterior thigh. If tumor spreads from the local site, however, it may not be possible to remove all of the TNF producing tumor.

5. Risk from murine retrovirus. Exposure of the cancer patient to retrovirus could theoretically pose a risk of insertional mutagenesis. It should be emphasized, however, that careful tests will be conducted to assure that the patient is not exposed to replication competent virus. The retrovirus derived from the Moloney murine leukemia virus has been modified so that it no longer contains any intact viral genes and thus cannot produce the envelope proteins necessary to package its RNA into an intact infectious virus (36,37,41,43,44). To assemble the retrovirus, a retrovirus packaging cell line was used that contained a second defective retrovirus which expresses the viral structural proteins. This packaging cell line does not produce replication competent retrovirus because of multiple modifications made to the second retrovirus that prevent its replication, including removal of signals required for RNA encapsidation, reverse transcription, and integration (37). Multiple assays will be performed on the final producer cell line, the retroviral vector supernatant as well as on the TIL prior to infusion to insure that no replication competent virus is present. These tests will include S+L- assays including 3T3 amplification, PCR assays for the envelope gene, and assays for reverse transcriptase (42,42). Any supernatants or TIL with evidence of any replication competent virus will not be utilized. The 3T3 amplification and S+L- assays are thought to be capable of detecting a single replication competent viral particle per ml (41).

Prior safety studies have shown that exposure of primates to large infusions of infectious murine amphotrophic virus produce no acute pathologic effects (44). In a study of 21 primates receiving retroviral mediated gene-modified autologous bone marrow cells no animal showed evidence of toxicity related to the gene transfer as long as 5 years after infusion (45), unpublished data).

It should be emphasized, however, that tumor will be transduced with the retroviral vector supernatant and then the tumor will be washed extensively and then grown for several weeks in the absence of supernatant. The tumor will then be washed extensively again prior to reinfusion into the patient and patients will thus not be exposed directly to the retroviral vector.

VII. Statistical Considerations.

Up to 14 patients with each type of cancer will be treated. If no responses are seen in these first 14 patients no further patients with that histologic type of cancer will be admitted to the protocol.